

PRMT5-Selective Inhibitors Suppress Inflammatory T Cell Responses and Experimental Autoimmune Encephalomyelitis

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In the autoimmune disease multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE), expansion of pathogenic, myelin-specific Th1 cell populations drives active disease; selectively targeting this process may be the basis for a new therapeutic approach. Previous studies have hinted at a role for protein arginine methylation in immune responses, including T cell-mediated autoimmunity and EAE. However, a conclusive role for the protein arginine methyltransferase (PRMT) enzymes that catalyze these reactions has been lacking. PRMT5 is the main PRMT responsible for symmetric dimethylation of arginine residues of histones and other proteins. PRMT5 drives embryonic development and cancer, but its role in T cells, if any, has not been investigated. In this article, we show that PRMT5 is an important modulator of CD4⁺ T cell expansion. PRMT5 was transiently upregulated during maximal proliferation of mouse and human memory Th cells. PRMT5 expression was regulated upstream by the NF- κ B pathway, and it promoted IL-2 production and proliferation. Blocking PRMT5 with novel, highly selective small molecule PRMT5 inhibitors severely blunted memory Th expansion, with preferential suppression of Th1 cells over Th2 cells. In vivo, PRMT5 blockade efficiently suppressed recall T cell responses and reduced inflammation in delayed-type hypersensitivity and clinical disease in EAE mouse models. These data implicate PRMT5 in the regulation of adaptive memory Th cell responses and suggest that PRMT5 inhibitors may be a novel therapeutic approach for T cell-mediated inflammatory disease. *The Journal of Immunology*, 2017, 198: 1439–1451.

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS that affects 2 million young adults worldwide (1). MS is driven by myelin-reactive inflammatory T cells, resulting in axonal demyelination and disability (2). The reactivation and expansion of myelin-specific inflammatory T cells is associated with active MS disease, including relapses (3–8). Therefore, drugs that suppress these processes may prevent or curtail the spread of this devastating disease.

MS is associated with increased Th1 and Th17 inflammatory responses (9) and deficient Th2 and regulatory T cell responses (10). In particular, an imbalance between reciprocal Th1 and Th2 responses was reported to be an important etiologic factor in MS.

Several studies showed that T cells from MS patients favor the proinflammatory Th1 phenotype as opposed to a Th2 phenotype (11–13). Furthermore, although myelin-reactive T cells are present in healthy individuals, MS patients have increased frequencies of myelin-specific T cells with an activated memory phenotype (14–16).

Upon re-exposure to Ag, memory T cells multiply quickly, providing a large army of responding T cells. TCR stimulation results in the activation of several signaling pathways, including the Notch, c-Myc, NFAT, ERK, JNK, NF- κ B, and mTOR pathways (17). Nuclear translocation of NFAT, Oct, NF- κ B and AP-1 transcription factors activate transcription of the pro-proliferative cytokine IL-2 (18). In addition, Notch and c-Myc induce T cell

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Abbreviations used in this article: ADA, adenosine deaminase; AUC, area under the curve; Bay11, Bay 11-7082; CMP5, compound 5; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PRMT, protein arginine methyltransferase; SAM, S-adenosyl methionine; SDM, symmetric dimethylation; shRNA, short hairpin RNA; siRNA, small interfering RNA; Tg, transgenic.

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proliferation (19), whereas the mTOR pathway is essential for glucose metabolism in proliferating T cells (20). Memory T cells transition quickly from a nonproliferative resting state to maximal proliferation 2–4 d after Ag exposure. This is followed by a return to a resting state 7–10 d later (21). Although this process is essential in the immune response against bacterial and other infections, memory T cell expansion in response to self-antigens can be harmful, resulting in excessive inflammation and autoimmunity.

The role, if any, that arginine methylation plays in this process remains vastly unexplored. However, previous studies provide some clues for further investigation. A role for methylation in physiologic immune responses was first suggested by the clinical signs of a debilitating immunodeficiency observed in adenosine deaminase (ADA)-deficient patients (22, 23). In ADA-deficient cells, the accumulation of adenosine and deoxyadenosine inhibits *S*-adenosyl methionine (SAM)-dependent methylation reactions (23, 24). In particular, TCR/CD28-mediated proliferation and cytokine production are inhibited in ADA-deficient patients (22, 23). Similarly, global methyltransferase inhibitors were shown to have strong immunosuppressive properties and abrogate T cell-mediated autoimmunity (25–28). Methylthioadenosine, a naturally occurring metabolite of SAM, can act as a methylation inhibitor when present at high concentrations (27). It was recently shown that alterations in the tumor environment result in high tumoral methylthioadenosine levels that inhibit protein arginine methylation and suppress anticancer human T cell responses. Therefore, accumulating evidence hints that the T cell effects of global methyltransferase inhibition are due to inhibition of protein arginine methyltransferases (PRMTs) (29, 30). However, conclusive evidence demonstrating that PRMTs are responsible for T cell suppression, as well as a specific role for the main symmetric dimethylation (SDM) enzyme PRMT5, has been lacking.

PRMTs are a family of enzymes that catalyze arginine methylation of nucleosomal histones on chromatin and other proteins. Among PRMTs, type I PRMTs (PRMT1–4, PRMT6, and PRMT8) catalyze asymmetric dimethylation, whereas type II PRMTs (PRMT5 and PRMT9) catalyze SDM at the ω -NH₂ of arginine (31). Although PRMT9 can catalyze SDM of certain substrates, most SDM reactions of histones are catalyzed by PRMT5 (32). Until recently, PRMT7 was considered a type II PRMT, but it was reclassified as a monomethylating type III PRMT (33). The modifications catalyzed by PRMTs play a crucial role in a variety of cellular processes, from differentiation to signaling and proliferation (34). Among PRMTs, PRMT5 appears to play a particularly relevant role in the regulation of cell death and malignant transformation processes in mouse and humans (35–38). Indeed, PRMT5 is upregulated in various human lymphoid malignancies (39–41) and solid tumors (42–48), and it promotes cancer cell proliferation and survival (38, 46, 49, 50). This led to the development of selective PRMT5-inhibiting drugs, such as compound 5 (CMP5) and EPZ015666, as potential new therapies in cancer (43, 51, 52). Although PRMT5 is clearly involved in tumor growth and survival, its role in T cell responses and its impact on autoimmunity are unknown.

In this study, we set out to investigate the role of PRMT5 activity in the expansion of pathogenic Th1 cells that leads to MS. We found that transient PRMT5 upregulation in response to TCR engagement *in vitro* is conserved in mouse and human memory Th1 and Th2 CD4⁺ T cells. PRMT5 upregulation in Th1 cells required NF- κ B signaling, and inhibition of PRMT5 activity with PRMT5-selective inhibitors blunted IL-2 secretion and proliferative responses of memory Th cells. Interestingly, pathogenic Th1 cells were more sensitive to PRMT5 inhibition than were benign Th2 or

naive T cells, a desirable immunological profile for MS drugs. *In vivo*, treatment with the novel PRMT5 inhibitor HLCL65 suppressed Ag-specific T cell responses and inflammation in the delayed-type hypersensitivity (DTH) model of inflammation and the experimental autoimmune encephalomyelitis (EAE) model of MS. This is the first evidence, to our knowledge, that PRMT5 plays an essential role in pathogenic Th1 cell responses. Further, we describe novel potent PRMT5-selective inhibitors that may provide a novel therapeutic strategy for Th1-mediated inflammatory autoimmune disease.

Materials and Methods

Mice

B10.PL (Jackson Laboratory) and myelin basic protein (MBP)_{Ac1–11}-specific TCR-transgenic (Tg) mice [described by Goverman et al. (53)] were bred in specific pathogen-free conditions at The Ohio State University Laboratory Animal Resources. C57BL/6 and BALB/c mice were purchased from Taconic and the Jackson Laboratory, respectively. All animal procedures were approved under electronic Institutional Animal Care and Use Committee protocol number 2013A00000151.

Reagents

The PRMT5 inhibitor CMP5 was designed and synthesized at The Ohio State University as previously described (51). Briefly, the compound was designed to fit into the PRMT5 enzyme crystal structure, partially covering the binding pockets for both the methyl group donor, SAM, and the acceptor protein arginine group. Stock CMP5 was dissolved in DMSO vehicle at a concentration of 100 mM and further diluted to 25 μ M in DMSO for *in vitro* assays. PRMT5 inhibitor HLCL65 was dissolved in DMSO vehicle at a concentration of 50 mM and was further diluted for *in vitro* assays. Stock Bay 11-7082 (Bay11) was dissolved in DMSO vehicle at a concentration of 10 mM and was further diluted \geq 1:1000 for *in vitro* assays.

HLCL65-binding interaction prediction

The crystal structure of human PRMT5:MEP50 complex (PDB ID: 4GQB) was used to predict the binding interaction of HLCL65 within the PRMT5 active site. The cocrystallized SAM analog (A9145C) and the histone H4-derived substrate peptide were deleted from the binding site. The small molecule ligand HLCL65 was prepared by Maestro (Schrodinger). Molecular docking was accomplished by AutoDock 4. The binding energy of the protein–ligand interaction in the shown binding mode was -13.14 kcal/mol.

Histone methyltransferase assays

Histone methylation was performed using 2 μ g of HeLa S3 core histones in the presence or absence of 15 μ l of affinity-purified human SWIth/sucrose nonfermentable-associated Flag-tagged PRMT5 or Flag-tagged PRMT7, as described previously (54). Reaction mixtures were spotted on Whatman P-81 filter paper and washed five times with 10 ml of 0.1 M sodium carbonate buffer (pH 9) to remove unincorporated [³H]SAM, and methylated peptides were detected by scintillation counting.

Cells

Mouse Th1 and Th2 cell lines were generated from MBP TCR-Tg mice (53). Naive T cells were isolated from TCR-Tg splenocytes by magnetic bead sorting using the CD4⁺CD62L⁺ T Cell Isolation Kit, mouse (Miltenyi Biotec) and activated with MBP_{Ac1–11} (0.5 μ g/ml) presented by irradiated splenocytes under Th1 (IL-12 + IFN- γ , anti-IL-4) or Th2 (IL-4, anti-IL-12 and anti-IFN- γ) conditions for two rounds. Th cell lines are not transformed and, therefore, were maintained by stimulation with MBP_{Ac1–11} and irradiated splenocytes in the presence of rIL-2 every 7–10 d. T cells collected 7–10 d after activation with MBP_{Ac1–11} and irradiated splenocytes provided the resting Th cell condition. To avoid the presence of non-T cells in *in vitro* experiments, resting Th1 or Th2 cell lines were activated with anti-CD3/CD28 in the presence or absence of the PRMT5 inhibitors CMP5 or HLCL65 or DMSO as vehicle control for various lengths of time. Human Th1 and Th2 cells were generated by isolating CD4⁺ T cells with a CD4⁺ T Cell Isolation Kit (STEMCELL Technologies) from human whole blood leukocytes from normal donors and activating on anti-CD3/CD28 Dynabeads (Thermo Fisher) under Th1 or Th2 conditions (same as mouse) for 1 wk. Th cells were removed from the Dynabeads and reactivated with plate-bound anti-CD3 (1 μ g/ml for Th1, 5 μ g/ml for Th2) and soluble

CD28 (1 $\mu\text{g/ml}$ for Th1, 2 $\mu\text{g/ml}$ for Th2) for further experiments. Naive CD4⁺ T cells were isolated with the mouse naive CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) and activated with 5 $\mu\text{g/ml}$ coated anti-CD3 and 2 $\mu\text{g/ml}$ soluble anti-CD28. CCMCL1 cells are a mantle cell lymphoma cell line that was described previously (55).

Western blot

Cells were lysed in Passive Lysis Buffer (Promega) or RIPA buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate) containing protease inhibitors and phosphatase inhibitors (Thermo Fisher). Protein concentrations were determined using a NanoDrop 2000 or BCA assay (Thermo Fisher). Equal quantities of protein (5–10 μg) were separated on 10–14% SDS-PAGE gels and transferred to nitrocellulose or polyvinylidene difluoride membranes (162-0177; Bio-Rad). Membranes were incubated with rabbit anti-PRMT5 Ab (1:500, ab31751; Abcam), anti-PRMT1 Ab (CST 2449S; Cell Signaling Technology), anti-PRMT7 Ab (ab22110; Abcam), or mouse anti- β -actin Ab (1:20,000, A1978; Sigma) overnight at 4°C or for 3 h at room temperature. After incubation with HRP-conjugated anti-rabbit Ab (1:15,000, A0545) or anti-mouse Ab (1:20,000, A9044; both from Sigma), Western blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher), and the luminescent signal was captured on film and developed on a Konica-Minolta SRX-101A or digitally on a Fuji LAS-4000 Imaging System. After incubation with fluorescently labeled anti-rabbit or anti-mouse secondary Abs (Odyssey LI-COR), Western blots were imaged with Odyssey CLx. The Western blotting bands were analyzed by ImageJ (Bio-Arts, Fukuoka, Japan) or Image Studio software.

RNA isolation

Total RNA was isolated with a mirVana kit (Life Technologies), according to the manufacturer's instructions, and stored at -80°C until analysis. RNA concentration and quality were determined using a NanoDrop 2000.

Real-time PCR

RNA (300–1000 ng) from profiled samples were cDNA transcribed using random primers and Superscript II (Applied Biosystems); TaqMan quantitative real-time PCR was performed using *mTbx21* (Mm00450960_m1) and *mHPRT* (Mm0044968_m1) primer sets (Life Technologies), according to the manufacturer's instructions. Samples were cDNA transcribed using random primers and Superscript III (Applied Biosystems) of similar amplification efficiency for test and control genes. An initial denaturation step at 95°C for 10 min was followed by 40 cycles of denaturation at 95°C for 15 s and primer annealing/extension at 60°C for 60 s. Results were analyzed using the comparative Ct method.

Cytokine ELISA

Cytokines were detected in supernatants at various points poststimulation using a sandwich ELISA. Mouse IL-2 reagents were from BD, mouse IL-17 reagents were purchased from eBioscience (Capture: 14-7175-85, Detection: 13-7177-85), human IL-2 reagents were purchased from BioLegend (Capture: 500302, Detection: 517605), and recombinant human IL-2 was purchased from Miltenyi Biotec. ELISA was performed as previously described (12).

[³H]thymidine proliferation assay

Th1 and Th2 cell lines were plated on anti-CD3/CD28-coated wells (100,000–125,000 cells per well) and treated with CMP5 inhibitor, or vehicle control (DMSO), and/or increasing concentrations of IL-2. Two days after treatment, cells were pulsed with 1 μCi of tritiated thymidine (³H]thymidine). After 18 h, cells were harvested on a Filtermate 196 harvester, and the amount of [³H]thymidine incorporated into the DNA was measured using a TopCount microplate scintillation and luminescence counter (both from Packard/Perkin-Elmer, Waltham, MA).

Intracellular flow cytometry

On collection day, cells were treated with PMA/ionomycin and GolgiStop (BD Biosciences) for 4–6 h and washed with FACS buffer prior to Fc region blockade and surface Ab staining (10 min, 4°C). Samples were fixed with Fixation/Permeabilization buffer and washed with Perm/Wash Buffer (554715; BD Biosciences). The intracellular proteins T-bet (644807; BioLegend), IL-17 (506916; BioLegend), and ROR γ t (12-698880; eBioscience) were stained with the corresponding Abs (T-bet clone: 4B10, IL-17 clone: TC11-18H10.1, and ROR γ t clone: AFKJS-9) for 30 min at 4°C . T-bet⁺ROR γ t⁺, IL-17⁺ROR γ t⁺, and T-bet⁺IL-17⁺ double-positive populations were analyzed within the CD4⁺CD44⁺ T cell gate.

Short hairpin RNA lentivirus transfection and transduction

Lentiviral vectors expressing five PRMT5-targeted short hairpin RNAs (shRNAs; target set RHS4533-EG10419) and the universal negative control, pLKO.1 (RHS4080), were acquired from Open Biosystems. HEK293T cells (Takara Clontech) were transfected with lentiviral vectors plus DNA vectors encoding HIV Gag/Pol and VSV-G in 10-cm dishes with Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions. Lentiviral particle-containing supernatant was collected after 72 h, filtered through 0.45- μm filters, and concentrated using ultracentrifugation in a Sorvall SW 41 swinging bucket rotor. Human Th1 cells were prepared by resuspending 500,000 cells in 50 μl of concentrated lentivirus plus 8 $\mu\text{g/ml}$ Polybrene. Human Th1 cells were transduced by spinoculation at $2000 \times g$ for 2 h at room temperature and incubated for 1 h at 37°C . Virus was washed out, and cells were plated on anti-CD3/CD28-coated plates for proliferation or protein.

Small interfering RNA transfection with Neon electroporation

To knockdown PRMT5, we selected three small interfering RNAs (siRNAs) targeting different areas in the PRMT5 gene. Two siRNAs (si#1: 5'-AAT TCC AAG GTG CAA TAG CGG CCT GTC TC-3' and si#2: 5'-ACA CUU CAU AUG UCU GAG A-3') were synthesized in-house with the Silencer siRNA Construction Kit (AM1620M; Thermo Fisher). The third siRNA (si#3) was purchased from Ambion (cat. no. s77695). For siRNA transfection, the Neon Transfection System (Invitrogen) was used, following the manufacturer's instructions and adapting them as indicated below. Human Th cells were prepared by washing twice with PBS, removing all of the supernatant after the last wash. Five million primary T cells were resuspended in 100 μl of T buffer containing 1.5 μg of siRNA and electroporated (one pulse at 2100 V for 20 ms) using a Neon 100- μl transfection pipette. Cells were mixed 1:1 with 100 μl of 2% Viability Buffer (a gift from Dr. R. Han, The Ohio State University) to promote cell viability after electroporation. Finally, cells were plated into media lacking penicillin/streptomycin. Proliferation and protein expression were monitored as indicated.

OVA-induced DTH

CFA (Difco) and OVA emulsion was prepared at a 1:1 v/v ratio for a final concentration of 1500 μg of OVA/1 ml of PBS. BALB/c mice were injected with 100 μl of emulsion in the dorsal proximal scruff and the base of the tail (150 μg of OVA per mouse). Control groups included nonimmunized mice and immunized mice that were not subsequently challenged with OVA. One week after immunization, aggregated OVA was prepared by suspending in PBS at a concentration of 10 mg/ml in a 15-ml tube. Solution was heated in an 80°C water bath for 60 min. Mice were challenged with 300 μg of aggregated OVA by injecting 30 μl of solution into the left footpad of immunized mice. After an additional week, mice were rechallenged in the same manner (nonimmunized mice were also challenged at this step). Twenty-four hours after the second challenge, mice were euthanized by CO₂ asphyxiation and cervical dislocation. Each footpad was measured using calipers for swelling (pre-euthanasia) and weighed for changes in mass. Additionally, spleens were removed and processed for in vitro studies.

Experimental autoimmune encephalomyelitis

For induced EAE, commercial Hooke Reagent or myelin oligodendrocyte glycoprotein (MOG; CS Bio) and CFA (Difco) emulsion were used. CFA/MOG emulsion was prepared in a 1:1 v/v ratio for a final concentration of 1000 μg MOG/1 ml of PBS. C57/B6 mice (Taconic) received 100 μl of emulsion s.c. in the dorsal proximal scruff and the base of the tail. About 2 h after immunization, mice were injected i.p. with 100 μl of 2 ng/ μl pertussis toxin. Twenty-four hours later, mice were injected again with 100 μl of 2 ng/ μl pertussis toxin. Mice were monitored for disease every day and treated with 25 mg/kg HLCL65 or DMSO vehicle control. At the indicated time points, mice were euthanized by injection with 20 mg/ml ketamine and 4 mg/ml xylazine (120 μl /20 g mouse) and perfused with PBS. Spleens, brains, and spinal cords were collected from representative mice and processed for in vitro studies. To isolate brain and spinal cord mononuclear cells, brains and spinal cords were processed through a 70- μm strainer and separated by a 70–30% isotonic Percoll gradient.

For spontaneous EAE, three MBP_{Ac1-11} TCR-Tg mice that developed EAE spontaneously (scores = 1.5–2) were euthanized by CO₂ asphyxiation and cervical dislocation. Splenocytes were isolated and activated with 2 $\mu\text{g/ml}$ MBP_{Ac1-11} for 48 h in the presence of PRMT5 inhibitors or vehicle control. T-bet, IL-17, and ROR γ t expression was analyzed by intracellular flow cytometry.

Results

PRMT5 protein is upregulated upon memory T cell reactivation

PRMT5 is overexpressed in several lymphoid malignancies, where it promotes uncontrolled cell growth and survival of transformed cells (35). However, its role in nonmalignant memory T cell proliferative responses is unknown. After exposure to their cognate Ag, previously sensitized T cells activate a signaling cascade that enhances metabolic activity and drives maximum proliferation at 2–3 d postactivation. Subsequently, the proliferative rate of T cells gradually decreases, and cells that survive the contraction period return to a nonproliferative resting state 7 d after activation (21). To determine whether PRMT5 plays a role in this process, PRMT5 expression was analyzed by Western blotting at various time points after MBP_{Ac1-11} TCR-Tg mouse memory Th1 or Th2

cells (characterized in Supplemental Fig. 1) were restimulated with immobilized anti-CD3/CD28. Compared with resting memory T cells, PRMT5 was upregulated 2.5-fold in Th1 cells and 2.4-fold in Th2 cells at the 48-h time point, the peak of PRMT5 expression (Fig. 1A, 1B). PRMT5 was subsequently downregulated at day 4, reaching baseline levels by day 7. These results led us to hypothesize that PRMT5 promotes proliferation during the normal cycle of T cell activation.

Selective PRMT5 inhibition blunts TCR-mediated memory T cell expansion

To determine whether PRMT5 activity is required for memory T cell proliferation, resting memory Th1 and Th2 T cells were activated in vitro, and the extent of T cell expansion was measured by [³H]thymidine-incorporation assay in the presence of the

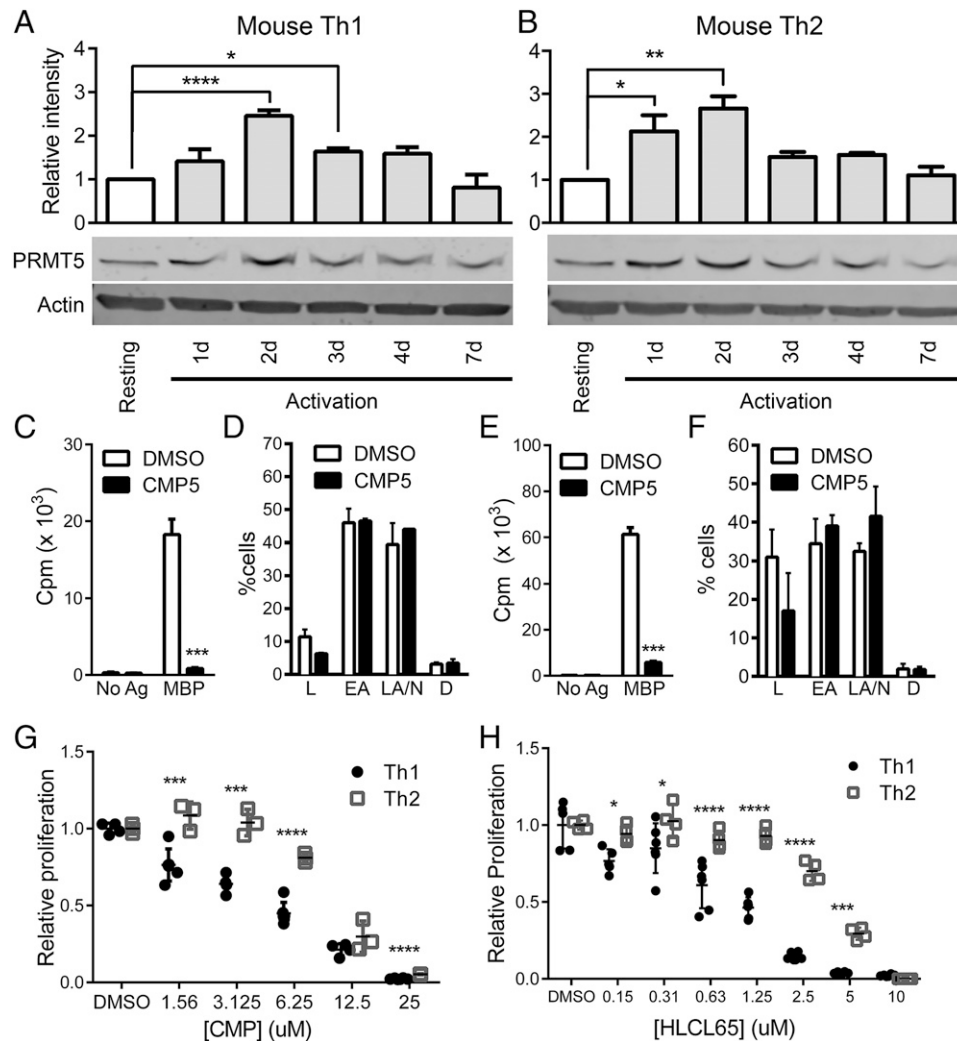


FIGURE 1. Proliferating murine Th1 and Th2 T cells express increased PRMT5 protein levels. MBP-specific TCR-Tg memory Th1 (**A**) and Th2 (**B**) cells were lysed at resting or at 1, 2, 3, 4, or 7 d after anti-CD3/CD28 stimulation and analyzed for PRMT5 protein expression by Western blotting. Resting cells were nonproliferating T cells and were collected 7–10 d after activation of the Th cell line with MBP_{Ac1-11} and irradiated splenocytes. β -actin was used as a loading control. Relative intensity quantification data are shown above a representative blot and were determined by normalizing PRMT5 expression to β -actin expression using Image Studio. Data are representative of three or four independent experiments ($n = 5$ for experiment shown). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, one-way ANOVA, followed by the Sidak multiple-comparison adjusted t test. MBP TCR-Tg memory Th1 (**C** and **D**) and Th2 (**E** and **F**) cells were stimulated with MBP_{Ac1-11} for 48 h in the presence of the PRMT5 inhibitor CMP5 or vehicle control (DMSO), and proliferation (**C** and **E**) and viability (**D** and **F**) were measured by [³H]thymidine incorporation or annexin V staining, respectively. Data are representative of three or four experiments ($n = 4$ for shown experiment) and are pooled from two independent experiments ($n = 4$). *** $p < 0.001$, Student t test. MBP TCR-Tg memory Th1 and Th2 cells were stimulated with anti-CD3/CD28 for 48 h in the presence of various concentrations of the PRMT5 inhibitors CMP5 (**G**) or HLCL65 (**H**) or vehicle control. Proliferation was monitored via [³H]thymidine incorporation. Data representative of three to four independent experiments (shown experiment $n = 4$). Plot error bars show \pm SD. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA, followed by the Sidak multiple-comparison adjusted t test. D, dead; EA, early apoptotic; L, live; LA, late apoptotic; N, necrotic.

Table I. IC₅₀ values for PRMT5 inhibitors CMP5 and HLCL65 in mouse and human Th1 and Th2 cells

Compound	Cell Type	IC ₅₀ (μM)	Hill Slope	R ²
CMP5	mTh1	3.7	-0.8334	0.96
	mTh2	9.2	-3.174	0.98
	hTh1	26.9	-3.454	0.94
	hTh2	31.6	-6.276	0.96
HLCL65	mTh1	1.1	-1.392	0.91
	mTh2	4	-2.321	0.97
	hTh1	5.7	-3.291	0.98
	hTh2	14.3	-4.15	0.97

h, human; m, mouse.

previously described PRMT5 inhibitor, CMP5 (51), or DMSO vehicle control. CMP5 was designed to selectively and reversibly bind within the PRMT5 active site to prevent transfer of the methyl group from the donor, SAM, to the arginine substrate-

binding pocket. CMP5 selectively inhibits PRMT5-mediated SDM but not other PRMTs (51). Overall, these data indicate that CMP5 is a selective PRMT5 inhibitor. CMP5 treatment of mouse Th1 and Th2 cells strongly inhibited T cell proliferation (Fig. 1C, 1E). A detailed analysis of apoptosis status via annexin V/propidium iodide staining revealed that there were no significant differences in apoptotic or dead cells in cells treated with PRMT5 inhibitor (Fig. 1D, 1F), indicating that the reduced proliferation could not be explained by cell death. Interestingly, we noticed a small, but significant, difference in Th1 versus Th2 suppression with CMP5. Th1 cell proliferation was more sensitive to PRMT5 inhibitors than was Th2 cell proliferation (95.4 versus 90.5% inhibition, respectively, $p < 0.005$, t test). To further explore this phenomenon, we analyzed the IC₅₀ values of the PRMT5 inhibitor CMP5 for both cell types. Indeed, we confirmed that CMP5 inhibited Th1 cell proliferation (IC₅₀ = 3.7 μM) more potently than Th2 cell proliferation (IC₅₀ = 9.2 μM) (Fig. 1G,

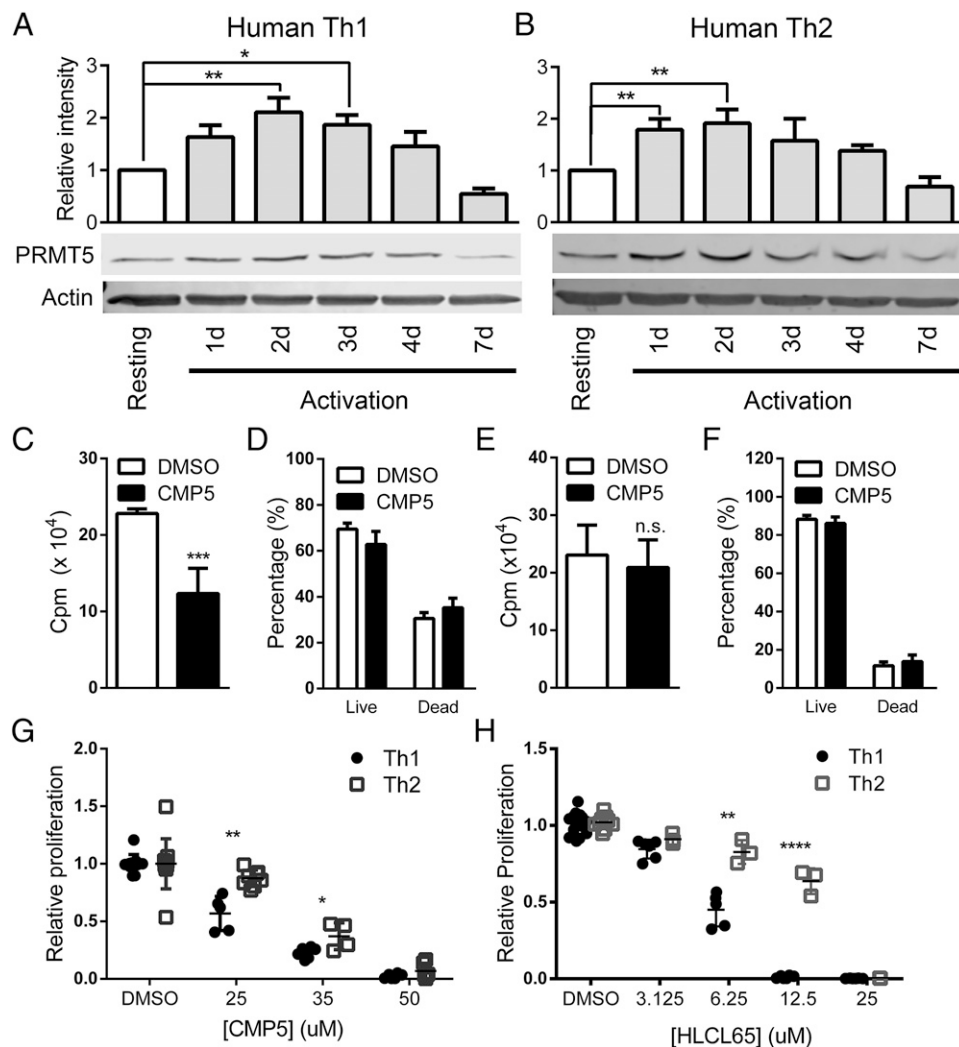


FIGURE 2. PRMT5 is essential for human Th1 and Th2 cell expansion. Human CD4⁺ T cells were isolated from whole blood and differentiated under Th1- or Th2-inducing conditions. After differentiation, Th1 (A) and Th2 (B) cells were reactivated on anti-CD3/CD28, and cells were lysed at rest and at 1, 2, 3, 4, and 7 d. PRMT5 protein expression was analyzed by Western blotting; β-actin was used as a loading control. Relative intensity quantification data are shown above a representative blot. Data are representative of three independent experiments ($n = 3$ for experiment shown). * $p < 0.05$, ** $p < 0.01$, one-way ANOVA, followed by the Sidak multiple-comparison adjusted t test. Human memory Th1 (C and D) and Th2 (E and F) T cells were activated with anti-CD3/CD28 for 48 h in the presence of the PRMT5 inhibitor CMP5 or vehicle control (DMSO), and the extent of T cell expansion (C and E) or viability (D and F) was measured by [³H]thymidine incorporation or trypan blue exclusion, respectively. Data are representative of three or four experiments ($n = 3$ for experiment shown). *** $p < 0.001$, Student t test. Human memory Th1 and Th2 cells were activated as in (C)–(F) in the presence of varying concentrations of vehicle control, CMP5 (G), or HLCL65 (H), and T cell proliferation was measured by [³H]thymidine incorporation. Plot error bars show \pm SD. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, two-way ANOVA, followed by the Sidak multiple-comparison adjusted t test. n.s., not significant.

Table I). To determine whether this phenomenon was isolated or could be replicated with CMP5 derivatives, we took advantage of a second-generation bioavailable PRMT5-selective inhibitor, HLCL65 (Supplemental Fig. 2A, 2B). HLCL65 selectively inhibited PRMT5-mediated SDM (Supplemental Fig. 2C, 2D). HLCL65 inhibited T cell proliferation more potently than CMP5, and it suppressed Th1 cells ($IC_{50} = 1.1 \mu\text{M}$) more effectively than Th2 cells ($IC_{50} = 4.0 \mu\text{M}$) (Fig. 1H, Table I). Overall, these data indicate that PRMT5 promotes murine memory T cell expansion and that inflammatory memory Th1 cells are more sensitive to targeting with PRMT5 inhibitors than are Th2 cells. To determine whether the proliferation or differentiation of newly activated naive T cells was similarly dependent on PRMT5 activity, we treated freshly isolated naive $CD4^+$ T cells with PRMT5 inhibitors. CMP5 and HLCL65 suppressed T cell proliferation in a dose-dependent manner (Supplemental Fig. 3A, 3B). Interestingly, and reminiscent of Th2 cells' behavior, naive $CD4^+$ T cells were more resistant than memory Th1 cells to PRMT5 inhibitors (Supplemental Fig. 3A, 3B). We also observed decreased $IFN-\gamma$ in supernatants from naive T cell cultures differentiated with CMP5 and HLCL65 in the absence of exogenous polarizing signals (Supplemental Fig. 3C, 3D). Overall, these results show prefer-

ential suppressive effects of PRMT5 inhibitors on memory Th1 responses that drive inflammatory autoimmune diseases, such as MS.

PRMT5 is essential for human T cell activation and expansion

Targeting of pathogenic Th cell expansion may be beneficial as a therapy in human Th1-mediated autoimmune diseases. To determine whether PRMT5 plays a similar role in human T cells, we restimulated previously differentiated human memory Th1- and Th2-enriched cells from healthy donors (characterized in Supplemental Fig. 4) and analyzed PRMT5 expression by Western blot from 0 to 7 d. We found that, similar to mouse T cells, PRMT5 was upregulated 2.1-fold in Th1 cells and 1.9-fold in Th2 cells by 48 h postactivation (Fig. 2A, 2B), and was subsequently downregulated to resting levels by 7 d postactivation. Importantly, PRMT5 was also essential for the expansion of human T cells. The PRMT5-selective inhibitor CMP5 preferentially suppressed the proliferation of human Th1 cells over Th2 cells (43 versus 9% inhibition, respectively, $p < 0.05$) (Fig. 2C, 2E), but it had minimal effects on cell death (Fig. 2D, 2F). To further evaluate the increased sensitivity of Th1 cells over Th2 cells to PRMT5 inhibition, we calculated the IC_{50} of human T cells when treated with

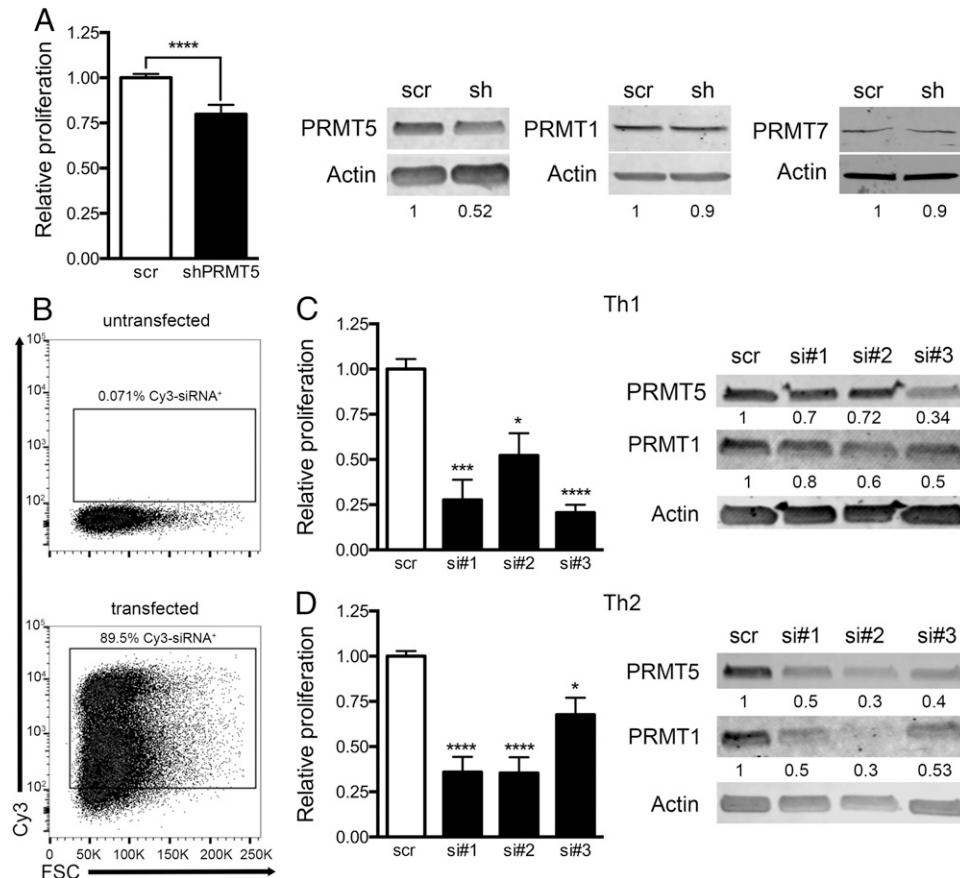
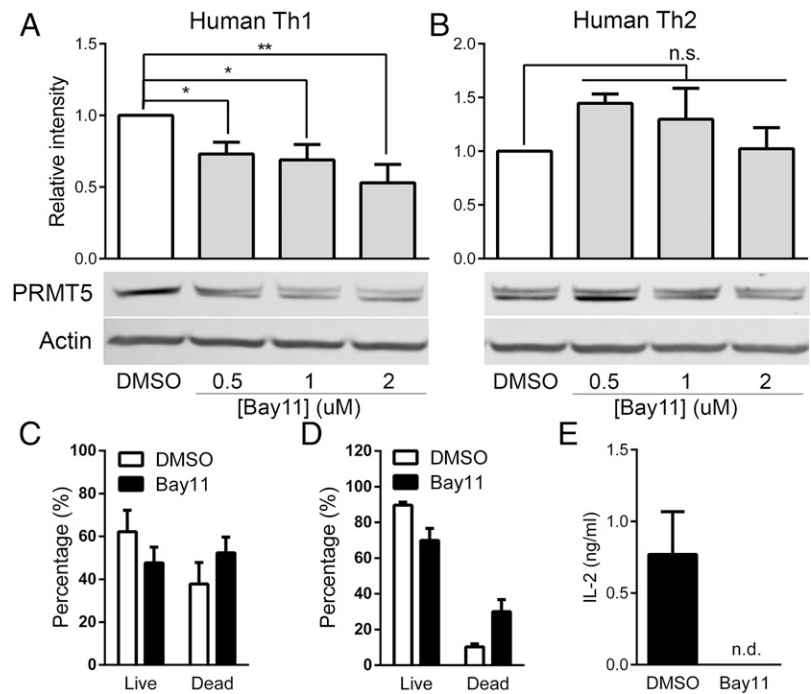


FIGURE 3. PRMT5 knockdown suppresses human T cell proliferation. (A) Human memory Th1 cells were activated with anti-CD3/CD28 for 48 h in the presence of PRMT5 shRNA or scrambled (scr) control, and proliferation was monitored via [^3H]thymidine incorporation. Proliferation relative to the scrambled control condition (left panel) (data pooled from three independent experiments, $n = 8$). PRMT5, PRMT1, and PRMT7 expression measured by Western blot and quantified using Image Studio software (right panels). **** $p < 0.0001$, Student t test. (B–D) Human Th1 and Th2 cells were activated as in (A) in the presence of a Cy3-labeled scramble control siRNA, scrambled siRNA control (scr), or three PRMT5-specific siRNAs (si#1 targets the 5' untranslated region, si#2 targets exon 15, and si#3 targets exon 3). (B) Cy3siRNA $^+$ cells (gated on $CD4^+$ cells) were quantified by flow cytometry as a measure of transfection efficiency. The transfection efficiency shown corresponds to Th1 cells. Equivalent efficiency was observed in Th2 cells. Proliferation of human Th1 (C) and Th2 (D) cells was monitored by [^3H]thymidine incorporation and expressed as a relative proliferation ratio to the NS transfection control condition. PRMT5 and PRMT1 protein expression was measured by Western blot and quantified using Image Studio software. Protein data are representative of three independent experiments and are pooled from three independent experiments ($n = 9$ for experiment shown). Plot error bars show \pm SEM. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA, followed by the Sidak multiple-comparison adjusted t test.

FIGURE 4. T cell activation drives PRMT5 expression in an NF- κ B-dependent manner. Differentiated human Th1 (A) or Th2 (B) cells were reactivated on anti-CD3/CD28 and treated with DMSO vehicle or increasing amounts of the NF- κ B pathway inhibitor Bay11 for 8 h. Cells were lysed for Western blot analysis of PRMT5 expression 48 h after initial activation. β -actin was used as a loading control. Relative intensity quantification data are shown above a representative blot quantified with Image Studio. Data are representative of three independent experiments ($n = 3$ for experiment shown). Differentiated human Th1 (C) and Th2 (D) cells were treated as described in (A) and (B), and viability was monitored by trypan blue exclusion. Data were pooled from three independent experiments ($n = 6$). (E) Differentiated human Th1 cells were treated as described in (A), and supernatants were collected to analyze the concentration of IL-2 production by ELISA. Data are representative of three independent experiments ($n = 2$). Plot error bars show \pm SD. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA, followed by the Sidak multiple-comparison adjusted t test. n.d., not detected; n.s., not significant.



CMP5 (Th1 $IC_{50} = 26.9 \mu M$ versus Th2 $IC_{50} = 31.6 \mu M$) or HLCL65 (Th1 $IC_{50} = 5.7 \mu M$ versus Th2 $IC_{50} = 14.3 \mu M$) (Fig. 2G, 2H, Table I). To genetically validate a role for PRMT5 in

proliferation, we knocked down PRMT5 expression via shRNA lentiviral transduction in human Th1 cells. PRMT5 shRNA partially reduced PRMT5 protein levels (45%) but not other PRMTs (PRMT1 and PRMT7), and it significantly decreased Th1 cell proliferation (Fig. 3A). Although significant, the mild suppression of proliferation could be explained by the low lentiviral transduction efficiency expected in primary T cells, coupled with the proliferative advantage of untransduced (i.e., PRMT5-sufficient) cells. To increase efficiency, we used the Neon Transfection System, which provided 70–90% transfection efficiency in our primary human Th1/2 cell lines (Fig. 3B). Electroporation with any of three PRMT5-specific siRNAs efficiently suppressed human Th1 and Th2 cell proliferation to a degree that correlated with PRMT5 knockdown (Fig. 3C, 3D and a positive Pearson correlation analysis of relative proliferation versus PRMT5 expression in Th1 and Th2 cells, $r = +0.7481$, $p < 0.0001$, data not shown). These results validate a role for PRMT5 in T cell-proliferative responses. Interestingly, we observed that PRMT5 siRNA transfection was accompanied by PRMT1 protein suppression, particularly in Th2 cells (Fig. 3C, 3D). Because the three siRNAs target different areas in PRMT5 (5' untranslated region, exon 3, or exon 15), and each one was confirmed to lack complementarity with PRMT1, it is unlikely that this is a direct effect of the siRNAs on PRMT1 but rather is an indirect effect via PRMT5. Further studies should aim to elucidate the significance of this finding. Overall, these data indicate that PRMT5 is functionally conserved in mouse and human T cells and plays a critical role in memory Th cell reactivation and expansion.

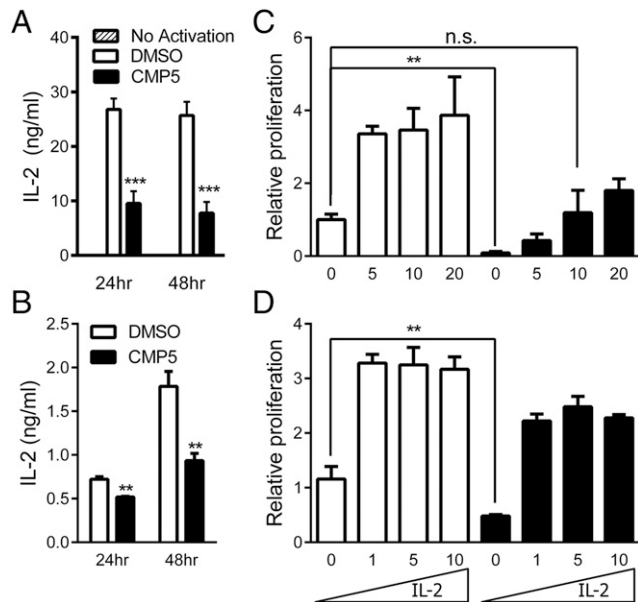


FIGURE 5. PRMT5 inhibition suppresses IL-2 production, and exogenous IL-2 rescues CMP5-inhibited Th cell proliferation. IL-2 measured by ELISA in supernatants of murine MBP TCR Th1 memory T cells (A) or human Th1 cells (B) stimulated through the TCR at various time points in the presence of CMP5 or vehicle control. Th2 cells are not shown because they do not secrete IL-2. Data are from two or three independent experiments ($n = 4$). ** $p < 0.01$, *** $p < 0.001$, Student t test. Proliferation at 48 h, measured by [3 H]thymidine incorporation, of mouse memory MBP TCR-Tg Th1 (C) or human memory Th1 (D) T cells activated with anti-CD3/CD28 in the presence of CMP5 or vehicle control and increasing amounts of exogenous IL-2 (ng/ml). Data are representative of two or three independent experiments ($n = 3$ for experiment shown). Plot error bars show \pm SD. ** $p < 0.01$, ANOVA, followed by the Sidak multiple-comparison adjusted t test. n.s., not significant.

PRMT5 expression is dependent upon TCR-induced NF- κ B signaling

The NF- κ B pathway was associated with PRMT5 in hematologic malignancies (56), but the pathways involved in PRMT5 expression in T cells are unknown. T cell reactivation activates several signaling pathways, including the NF- κ B pathway, leading to IL-2 production and proliferation. NF- κ B transcription factors are kept inactive in the cytoplasm through binding to the inhibitory I κ B subunits. Upon T cell activation, the I κ B subunit is phosphorylated by IKK α and proteasomally degraded, allowing nuclear

translocation of NF- κ B and activation of transcription (57). To test whether NF- κ B played a role in the upregulation of PRMT5 expression after TCR/CD28 costimulation, we treated human Th cells with the IKK- α inhibitor Bay11 during the first 8 h of activation. After 48 h of TCR/CD28 stimulation, we observed a decrease in PRMT5 protein levels in healthy human donor Th1 cells treated with Bay11 (Fig. 4A). In contrast, PRMT5 expression in Th2 cells was less dependent on NF- κ B signaling than was that in Th1 cells, as evidenced by stable levels of PRMT5 expression in Th2 cells treated with Bay11 (Fig. 4B). There were no differences in cell death with Bay11 treatment (Fig. 4C, 4D), indicating that the changes in protein expression observed could not be explained by cell death. As expected (58), NF- κ B inhibition also resulted in a 65% reduction in downstream cytokine IL-2 levels in Th1 cells (Fig. 4E). These data are consistent with NF- κ B signaling promoting PRMT5 expression and IL-2 secretion during TCR-mediated activation of Th1 and Th2 cells.

PRMT5 inhibition suppresses IL-2 secretion, and IL-2 restoration rescues T cell expansion

TCR-mediated activation of the NF- κ B pathway promotes IL-2, an important pro-proliferative T cell cytokine. We observed reduced IL-2 with Bay11-mediated suppression of PRMT5, and PRMT5 was linked to IL-2 production in Jurkat cancer T cells (59). Therefore, we explored whether PRMT5 inhibition affected IL-2 secretion in reactivated mouse or human Th1 and Th2 memory T cells. Vehicle-treated mouse (Fig. 5A) and human (Fig. 5B) Th1 cells secreted high levels of IL-2 at 24 and 48 h postactivation. In contrast, and as described previously for Th2 cells (60), no IL-2 was detected in Th2 cell su-

pernatants (data not shown). PRMT5 inhibition resulted in a reduction in IL-2 secretion that ranged from 50 to 75% for mouse Th1 cells and from 30 to 80% for human Th1 cells (Fig. 5A, 5B). These data suggested that loss of IL-2 secretion may contribute to the suppression of proliferation observed in Th1 cells treated with PRMT5 inhibitors. To test whether the blunted T cell proliferation observed after PRMT5 inhibition could be rescued with IL-2 supplementation, memory Th1 cells were activated with anti-CD3/CD28 in the presence or absence of PRMT5 inhibitor and with increasing amounts of exogenous IL-2. Doses from 1 to 20 ng/ml were chosen because it was calculated that ≥ 10 ng/ml would be needed to restore the supernatant IL-2 levels observed in the vehicle Th1 condition (as in Fig. 5A). T cell proliferation was evaluated using a tritiated thymidine-incorporation assay. Treatment with 25 μ M CMP5 inhibited mouse Th1 cell proliferation by 91% ($p < 0.001$, t test), and addition of IL-2 enhanced proliferation in the vehicle condition, reaching a peak at 5 ng/ml (Fig. 5C). Addition of IL-2 in the presence of PRMT5 inhibitor increased proliferation in a dose-dependent manner, reaching 100% of the control values at 10 ng/ml (Fig. 5C). Similarly, treatment with 25 μ M CMP5 suppressed human Th1 cell proliferation by 50%, and addition of exogenous IL-2 rescued proliferation (Fig. 5D). The recovery of Th1 T cell proliferation with exogenous IL-2 indicates that IL-2 pathways are active downstream of IL-2R and supports the notion that inhibition of IL-2 secretion by PRMT5 inhibitors contributes to the observed reduction in Th1 T cell proliferation. However, additional mechanisms may play a role in PRMT5 inhibitor-mediated suppression of proliferation, particularly in Th2 cells.

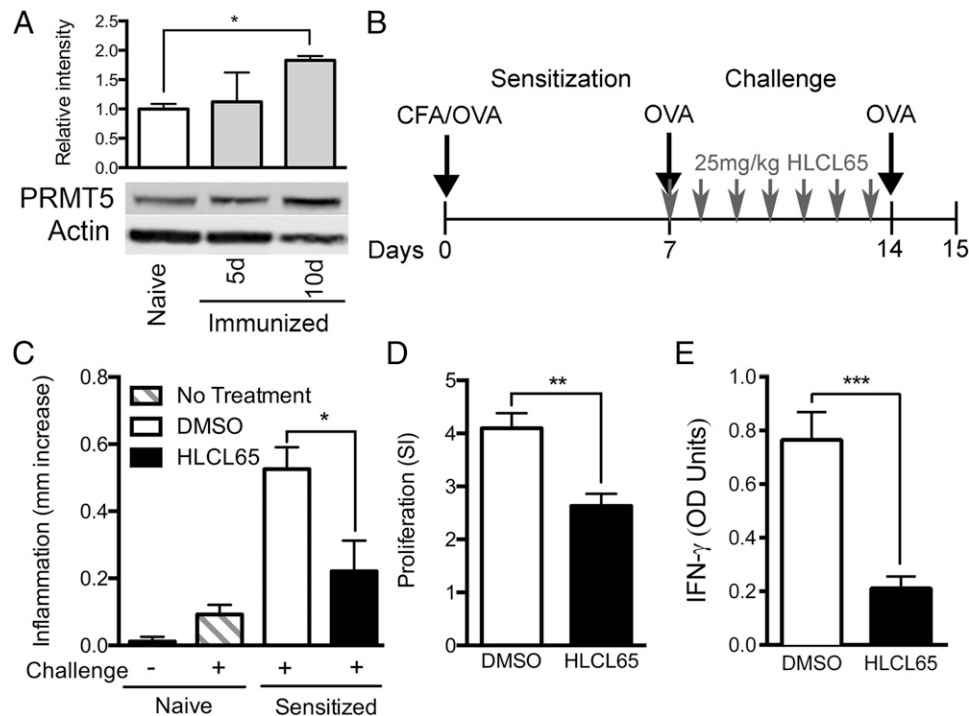


FIGURE 6. PRMT5 inhibition suppresses T cell responses and inflammation in the DTH model. **(A)** PRMT5 expression in spleens from naive mice or from mice 5 or 10 d after immunization with OVA and CFA. Spleens were crushed under liquid nitrogen and lysed for Western blot analysis using β -actin as a loading control. Data are representative of two independent experiments (data shown $n = 3$). **(B)** Schematic diagram of DTH model and treatment strategy. Mice were sensitized to OVA with CFA/OVA immunization (flanks and tail base); mice were challenged in the footpad with OVA 7 and 14 d later, and treated daily with 25 mg/kg PRMT5 inhibitor HLCL65 or DMSO i.p. between days 7 and 14. **(C)** Inflammation was evaluated on day 15 after initial sensitization using calipers to quantify footpad swelling. **(D)** Day-15 splenocytes were activated in the presence or absence of OVA for 72 h, and proliferation was monitored by [3 H]thymidine incorporation. **(E)** Supernatants were collected from splenocytes isolated as described in (C), and IFN- γ production was measured by ELISA ($n = 8$). Data are representative of two independent experiments. Plot error bars show \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

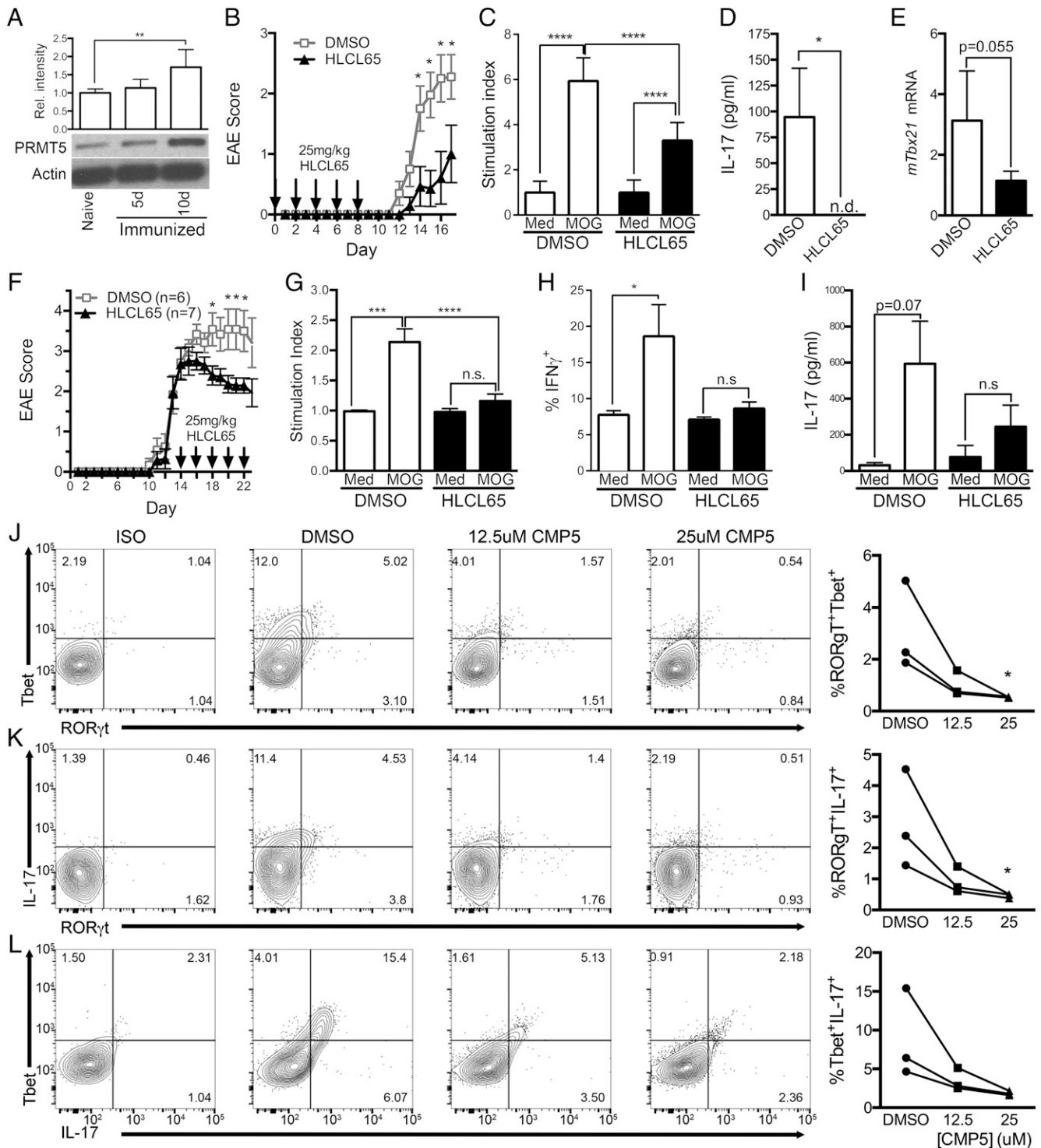


FIGURE 7. PRMT5 inhibition suppresses in vivo inflammatory T cell responses and clinical disease in the EAE murine model of MS. **(A)** PRMT5 expression in mouse spleens from naive mice or from mice 5 or 10 d after immunization with CFA/MOG in the preclinical EAE phase. Spleens were crushed under liquid nitrogen and lysed for Western blot analysis using β -actin as a loading control. Representative data of two independent experiments are shown ($n = 4$). **(B)** Clinical EAE score in mice preventatively treated with DMSO vehicle or 25 mg/kg HLCL65 (every other day; arrows indicate treatment) from days 0 to 9 after CFA/MOG immunization to induce EAE ($n = 10$). EAE score and the day of onset were monitored blindly daily. Mann-Whitney test. Splenocytes **(C)** and brain/spinal cord mononuclear cells **(D)** were isolated from DMSO- or HLCL65-treated mice and activated in the presence or absence of MOG35–55. **(C)** MOG-specific proliferation was monitored via [3 H]thymidine incorporation ($n = 3$). **** $p < 0.0001$, one-way ANOVA, followed by the Sidak multiple-comparison adjusted t test. **(D)** MOG-specific IL-17 production was measured by ELISA ($n = 2$ samples each pooled from six individual mice per group). * $p < 0.05$, Student t test. **(E)** RNA was isolated from brains and spinal cord homogenates of DMSO- or HLCL65-treated mice, and *Tbx21* mRNA expression was measured by quantitative real-time PCR (Student t test, $n = 2$ or 3 per group). **(F)** EAE in mice treated with DMSO vehicle or 25 mg/kg HLCL65 (every other day; arrows indicate treatment) starting on day 14 postimmunization, after EAE developed. Mice were randomly assigned to either group (pretreatment average scores were 1.92 for DMSO and 1.96 for HLCL65) and blindly scored for EAE daily ($n = 6$ –7). Mann-Whitney test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(G–I)** Mice were immunized with CFA/MOG and treated with DMSO or 25 mg/kg HLCL65 every other day starting 1 wk after immunization. Splenocytes were isolated from DMSO- or HLCL65-treated mice (*Figure legend continues*)

PRMT5 inhibition suppresses in vivo OVA-induced DTH inflammatory responses

The effectiveness of PRMT5 inhibitors at suppressing inflammatory memory T cell responses suggested that they may be beneficial in inflammatory or autoimmune disease. To test this, we used the OVA-induced DTH mouse model and HLCL65, a more potent and bioavailable derivative of CMP5 (Figs. 1H, 2H, Supplemental Fig. 2). First, we analyzed PRMT5 expression in the spleen of untreated mice after OVA immunization with CFA. We observed that, at 10 d after immunization, PRMT5 expression was upregulated significantly in the spleen (Fig. 6A), suggesting that PRMT5 expression is relevant to in vivo DTH immune responses. In the DTH model (outlined in Fig. 6B), OVA immunization with CFA induces an OVA-specific T cell response that causes footpad inflammation in mice upon subsequent exposure to adjuvant-free OVA and memory CD4⁺ T cell expansion. HLCL65 treatment during the rechallenge period reduced footpad swelling, a measure of inflammation, by 40% ($p < 0.05$, Fig. 6C). In addition, compared with vehicle, HLCL65 treatment reduced OVA-specific T cell proliferation by 36% (Fig. 6D) and IFN- γ production by 70% (Fig. 6E). These data indicate that our novel PRMT5 inhibitor HLCL65 suppresses T cell-mediated responses and inflammation in vivo.

Prophylactic or therapeutic treatment with HLCL65 ameliorates EAE

The ability of PRMT5 inhibitors to suppress in vivo inflammatory T cell responses could be beneficial in the autoimmune disease MS. We observed that PRMT5 was upregulated in the spleen at 5 and 10 d after immunization with CFA/MOG (Fig. 7A), suggesting that PRMT5 plays an important role in the immune response against myelin Ags in vivo. In the MOG-induced murine EAE model, T cell responses against this myelin Ag resulted in ascending paralysis. We first tested whether short-term prophylactic HLCL65 treatment (5 d, 25 mg/kg every other day starting at immunization) could prevent EAE. Indeed, HLCL65 treatment resulted in delayed disease onset (16.9 d for HLCL65-treated mice versus 13.4 d for vehicle-treated mice) and a 33% reduction in disease incidence compared with vehicle-treated mice (Table II). Importantly, HLCL65-treated mice presented reduced EAE disease burden, as measured by the area under the curve (AUC), compared with vehicle control (AUC HLCL65 versus vehicle: 2.1 versus 8.2, $p = 0.016$) (Fig. 7B, Table II). These clinical effects were associated with reduced MOG-specific T cell-proliferative responses (Fig. 7C), reduced CNS IL-17 production (Fig. 7D), and a trend toward suppressed *Tbet* mRNA expression in the CNS (Fig. 7E). With these promising results, we tested HLCL65 during a therapeutically relevant window for MS patients (i.e., after clinical signs had developed). HLCL65 treatment, beginning at 14 d after immunization (average score at treatment initiation = 2.7 out of 5), suppressed existing clinical signs of EAE, as measured by the total disease burden (AUC HLCL65 versus vehicle: 19.1 ± 1.6 versus 27.31 ± 3.1 , Fig. 7F, Table III). This disease suppression correlated with a reduction in MOG-specific T cell proliferation in HLCL65-treated mice (Fig. 7G). Additionally, inflammatory Th1 and Th17 responses were diminished in

HLCL65-treated mice (Fig. 7H, 7I). These data further support that PRMT5 activity may be essential for T cell function and that our novel PRMT5-selective inhibitors effectively suppress T cell-mediated inflammation. Next, to test whether PRMT5 inhibitors could suppress preformed encephalitogenic Th17 cells, splenocytes from MBP TCR-Tg mice that had spontaneously developed EAE (average score = 1.7) were activated with MBP_{Ac1-11} in the presence or absence of CMP5 and HLCL65. CMP5 treatment significantly suppressed the frequency of pathogenic T-bet⁺ROR γ t⁺ (61) (Fig. 7J) and classic IL-17⁺ROR γ t⁺ (Fig. 7K) Th17 cells in a dose-dependent manner. A similar dose-dependent decrease in pathogenic T-bet⁺IL-17⁺ Th17 cells (62) was apparent, but it did not reach statistical significance (Fig. 7L). Similar results were observed with HLCL65 treatment (data not shown). Taken together, these results indicate that PRMT5 promotes pathogenic Th1 and Th17 cell responses that lead to inflammation and autoimmunity.

Discussion

Memory T cell reactivation after Ag exposure rapidly induces T cell proliferation and effector function. This process can be beneficial, as in vaccination immunity, or deleterious, as in perpetuation of pathogenic responses in autoimmunity. In this article, we show by expression knockdown and pharmacologic means that PRMT5, a methyltransferase that catalyzes SDM of arginine residues in histones and other proteins, promotes the activation and expansion of memory Th lymphocytes following Ag re-exposure.

The first indications of a key role for arginine methylation in lymphocyte activation originated from conditions and treatments that inhibit all SAM-dependent methylation reactions (25, 26). PRMTs were proposed to mediate some of these effects (29), but the role of individual PRMTs in these processes remained unresolved. We found that Ag re-exposure in memory T cells upregulates PRMT5 expression as T cells proliferate and expand, followed by a contraction phase in which PRMT5 expression is progressively lost. The temporal link between PRMT5 expression and proliferation, together with the observed inhibition of proliferation upon selective PRMT5 inhibition, indicates that PRMT5 activity is necessary for TCR engagement-induced memory T cell expansion. Th2 cell expansion was less dependent on PRMT5 activity than was that of Th1 cells. This difference was reproduced in mouse and human Th cells, indicating that this is a conserved difference that may impact human disease. However, differential sensitivity to PRMT5 inhibition did not appear to stem from differences in PRMT5 expression, which was equivalent in Th1 and Th2 cells. It is possible that PRMT5 activity is lower in Th2 cells than in Th1 cells as a result of the expression of type I methyltransferases, which compete with PRMT5 for substrates (63). This difference offers the intriguing possibility that targeting PRMT5 may modulate the Th1/Th2 balance defect observed in autoimmune/inflammatory diseases, such as MS (12, 13).

The exact chain of events that leads to PRMT5 upregulation in T cells is unclear. A link between the NF- κ B pathway leading to activation of the repressive p65/HDAC/Sp1 complex and loss of PRMT5 targeting microRNA was reported in mantle cell

10 d after immunization and activated in the presence or absence of MOG. MOG-specific proliferation was monitored via [³H]thymidine incorporation (G), IFN- γ ⁺-secreting cells were quantified by flow cytometry (gated on CD4⁺, CD44⁺ cells) (H), and IL-17 production was measured by ELISA (I). * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA, followed by the Sidak multiple-comparison adjusted *t* test. (J-L) Splenocytes were isolated from MBP TCR-Tg mice with spontaneous EAE and activated with MBP_{Ac1-11} in the presence of the indicated concentrations of the PRMT5 inhibitors CMP5 and HLCL65 or DMSO vehicle control for 48 h. Frequencies of ROR γ t⁺T-bet⁺ (J), ROR γ t⁺IL-17⁺ (K), and IL-17⁺T-bet⁺ (L) T cells were quantified by intracellular flow cytometry on a CD4⁺CD44⁺ T cell gate. One-way ANOVA, followed by Sidak multiple-comparison adjusted *t* test. Plot error bars show \pm SD. * $p < 0.05$. n.d., not detected; n.s. not significant.

Table II. Prophylactic treatment with HLCL65 ameliorates EAE

Disease Parameter	DMSO	HLCL65	Change from DMSO	<i>p</i> Value
EAE incidence (%)	90	57	-33	n/a
Disease onset (d; mean ± SD)	13.44 ± 0.53	16.86 ± 0.96	+3.42 d	0.0053
AUC (mean ± SD)	8.214 ± 1.58	2.143 ± 1.41	-6.071	0.0159
Score on day 17 (mean ± SD)	2.3 ± 0.37	1.0 ± 0.48	-1.3	0.048
Maximum score (mean ± SD)	2.45 ± 0.37	1.0 ± 0.48	-1.45	0.0285
<i>n</i>	10	7	n/a	n/a

n/a, not applicable.

lymphoma (64). We also found previously that NF- κ B inhibition suppresses PRMT5 expression in EBV-transformed cells (51). Because TCR engagement activates the NF- κ B pathway in T cells, a similar mechanism may regulate PRMT5 expression in T cells. Indeed, blocking NF- κ B signaling attenuated, but did not completely eliminate, PRMT5 expression in human Th1 cells. This indicates that, although NF- κ B is an important driver of PRMT5 expression in Th1 cells, other TCR-induced pathways play a more significant role in regulating PRMT5 expression, especially in Th2 cells. TCR signaling cascades include the NFAT, ERK1/2, p38, and JNK MAPK pathways. Interestingly, inhibitors of the p38 and JNK MAPK pathways, but not the ERK1/2 pathway, were shown to inhibit hypoxia-induced upregulation of PRMT5 in lung epithelial cells (65). Although future studies are required to clarify the extent to which these pathways affect PRMT5 upregulation in T cells, NF- κ B appears to play a major role in TCR-induced PRMT5 expression in human Th1, but not Th2, cells. Additionally, several studies showed that PRMT5 activates NF- κ B signaling through arginine methylation of p65 (56, 66–68), suggesting that the NF- κ B–PRMT5 signaling axis could involve a positive-feedback loop. Additional studies are required to validate this feedback loop and evaluate its role in T cells.

Several pathways downstream of TCR activation converge upon activation of the IL-2 promoter to induce T cell proliferation (18, 19, 69). In this study, we found that IL-2 secretion is dependent on PRMT5 activity and that addition of exogenous IL-2 to PRMT5 inhibitor-treated cells restored proliferation in Th1 cells. A role for PRMT5 in IL-2 production is consistent with the observations of Richard et al. (59); they reported that PRMT5 siRNA suppresses IL-2 secretion in the Jurkat cancer T cell line. This effect is thought to be mediated by PRMT5-catalyzed arginine methylation on histones. In support of this hypothesis, symmetrically dimethylated proteins associate with the IL-2 promoter after T cell activation. In contrast, PRMT5 did not associate directly with the IL-2 promoter. These data are consistent with PRMT5 indirectly regulating IL-2 expression via SDM of target proteins. Although the specific proteins that are methylated and bind to the IL-2

promoter remain to be defined, two proteins that form an IL-2 promoter-binding complex, NF-45 and NF-90, were proposed as candidate targets (59). Another candidate is the TCR signaling protein Vav-1, whose SDM was reported to promote IL-2 expression (70). Overall, our data point to IL-2 as one of the mechanisms by which PRMT5 regulates proliferation in Th1 cells. However, because we observed only a 60% reduction in IL-2 production, yet T cell proliferation is reduced by 90–95% when treated with CMP5, it is likely that PRMT5 regulates proliferation by several mechanisms. Because Th2 cells do not secrete large amounts of IL-2, further studies are required to determine the mechanism by which PRMT5 promotes Th2 cell proliferation.

Memory T cell responses play a critical role in chronic T cell-mediated diseases, such as autoimmunity and allergy (71, 72). For example, increased memory T cells were found in MS patients with active disease, and they increased further during disease flare (4), whereas the memory/naive T cell ratio diminishes in patients responding to therapy (73). Importantly, inhibition of methyltransferases successfully suppresses T cell activation and established clinical EAE and other inflammatory/autoimmune diseases (25–28), but the lack of selectivity has prevented the development of these treatments as therapy. Our data indicate that selective PRMT5 inhibition reproduces the suppression of memory T cell expansion observed with pan-methyltransferase inhibitors and may be similarly effective in autoimmunity. Indeed, in vivo treatment with PRMT5 inhibitors suppressed two models of inflammatory/autoimmune disease: DTH footpad inflammation and EAE CNS inflammation. Importantly, clinically established EAE disease was responsive to PRMT5 inhibitor treatment. Our data are consistent with T cells being a major target of PRMT5 inhibitors in EAE, although we cannot rule out a clinical contribution of PRMT5 inhibition in non-T cells (CNS cells or APCs). Effects on APCs could result in reduced TCR engagement and T cell responses. However, in vitro experiments showed similar suppressive effects when T cells are activated by anti-CD3/CD28 (Fig. 1E) or Ag-loaded APCs (Fig. 1C). In addition, HLCL65 treatment of EAE suppressed previously generated memory T cell

Table III. Therapeutic treatment with HLCL65 ameliorates EAE

Disease Parameter	DMSO	HLCL65	Change from DMSO	<i>p</i> Value
EAE incidence (%): pretreatment	100	100	0	n/a
Disease onset (d): pretreatment (mean ± SD)	11.67 ± 0.49	12.43 ± 0.37	+0.76	NS
Score on day 14 (mean ± SD) ^a	2.7 ± 0.40	2.7 ± 0.40	0	NS
AUC: pretreatment (mean ± SD)	4.60 ± 1.15	3.91 ± 0.91	-0.69	NS
AUC: posttreatment (mean ± SD)	27.31 ± 3.13	19.11 ± 1.61	-8.2	0.0329
Maximum score: pretreatment (mean ± SD)	2.7 ± 0.40	2.7 ± 0.40	0	NS
Maximum score: posttreatment (mean ± SD)	3.88 ± 0.38	2.93 ± 0.44	-0.95	0.0535
Score on day 22 (mean ± SD)	3.5 ± 0.51	2.14 ± 0.19	-1.36	0.0228
<i>n</i>	6	7	n/a	n/a

^aTreatment initiation occurred on day 14.

n/a, not applicable.

responses, which are less dependent on APC costimulation. The similarity in the suppression of proliferation and inflammatory cytokines from in vitro and in vivo DTH/EAE studies is also consistent with T cells being a major target. To investigate relevance to human disease, we analyzed genome-wide association studies from the International Multiple Sclerosis Genetics Consortium and the Wellcome Trust Case Control Consortium. Interestingly, rs4410871 was identified as a high-frequency single-nucleotide polymorphism in the *MYC* locus in MS patients (74). *MYC* was shown to be upregulated after T cell activation (75) and to promote PRMT5 expression (37, 38). Taken together, these data suggest that PRMT5 could play a significant role in human disease.

In summary, this is the first report, to our knowledge, of the role of PRMT5 expression in in vitro and in vivo nonmalignant T cell responses. Our work identifies PRMT5 as an epigenetic modifier enzyme that promotes memory Th cell expansion. Memory T cell expansion of inflammatory Th1 cells and, to a lesser extent, Th2 cells, was dependent on PRMT5 activity. Finally, PRMT5 inhibitors suppressed T cell-mediated inflammatory and autoimmune disease, suggesting that PRMT5 may be a promising therapeutic target for autoimmune and other T cell-mediated diseases.

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Disclosures

R.A.B. and C.L. have a patent on PRMT5 inhibitors. M.G.-d.-A. has a patent pending. The other authors have no financial conflicts of interest.

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